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Synaptic release of GABA by AgRP neurons is required for normal regulation of energy balance

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Abstract

The physiologic importance of GABAergic neurotransmission in hypothalamic neurocircuits is unknown. To examine the importance of GABA release from agouti-related protein (AgRP) neurons (which also release AgRP and neuropeptide Y), we generated mice with an AgRP neuron-specific deletion of vesicular GABA transporter. These mice are lean, resistant to obesity and have an attenuated hyperphagic response to ghrelin. Thus, GABA release from AgRP neurons is important in regulating energy balance.

AgRP neurons release AgRP, neuropeptide Y (NPY) and GABA and are important in regulating body weight¹⁻³. However, the deletion of AgRP and/or NPY has little effect on body weight⁴. This raises the possibility that the release of GABA may be important^{2,5}. A direct GABAergic action from AgRP neurons to pro-opiomelanocortin (POMC) neurons has been suggested to mediate the ability of leptin to indirectly excite POMC neurons^{2,5-7}. However, because of the lack of methodological approaches, the physiologic effect of GABAergic neurotransmission in the hypothalamus is unknown.

Vesicular GABA transporter (VGAT, encoded by *Vgat*, also known as *Slc32a1*) is required for presynaptic release of GABA⁸. To disrupt GABA release in a neuron-specific manner, we generated *Vgat^{fllox/flox}* mice (Fig. 1a and Supplementary Methods online). To ensure the specificity of *cre* expression in AgRP neurons, we generated *Agrp-Ires-cre* knockin mice (Fig. 1b and Supplementary Methods). We confirmed the specificity of Cre activity by immunostaining for green fluorescent protein (GFP) in *Agrp-Ires-cre* mice that were crossed with mice bearing a Cre-dependent GFP reporter transgene (Z/EG mice)⁹ (Fig. 1c and Supplementary Methods). We generated mice that lack GABA release from AgRP neurons by crossing *Vgat^{fllox/flox}* mice (129, C57BL/6 background) with *Agrp-Ires-cre* mice (129, C57BL/6 background). The study subjects were littermate offspring of *Vgat^{fllox/flox}* mice and *Agrp-Ires-cre*; *Vgat^{fllox/flox}* mice. Deletion of VGAT in AgRP neurons by Cre recombinase was confirmed in *Agrp-Ires-cre*; *Vgat^{fllox/flox}*; Z/EG mice (Supplementary Fig. 1 online). To establish that GABA release from AgRP neurons was disrupted, we recorded inhibitory postsynaptic currents (IPSCs, measuring GABA-mediated current) in cultured AgRP neurons with autaptic synapses¹⁰. We detected IPSCs in 7 out of the 13 AgRP neurons recorded from control mice,

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AUTHOR CONTRIBUTIONS

Q.T. and B.B.L. conceived and designed the experiments. Q.T. generated the *Vgat^{fllox/flox}* mice and conducted the experiments. J.E.J. generated the *Agrp-Ires-cre* mice. C.-P.Y. carried out the electrophysiological recording studies. Q.T. and B.B.L. wrote the paper. J.K.E. provided guidance for many aspects of this study. All authors discussed the results and commented on the manuscript.

but only detected IPSCs in 0 out of 12 AgRP neurons derived from *Agrp-Ires-cre*; *Vgat^{flox/flox}* mice (Fig. 1d). Thus, deletion of *Vgat* prevents the synaptic release of GABA from AgRP neurons. We observed no difference in AgRP immunostaining patterns in *Agrp-Ires-cre*; *Vgat^{flox/flox}* mice, suggesting that there was no alteration in AgRP neuron development resulting from the disruption of GABA release (Supplementary Fig. 2 online).

Agrp-Ires-cre; *Vgat^{flox/flox}* mice showed reduced body weight on chow diet in both males (Fig. 2a) and females (Supplementary Fig. 3 online). We did not observe a change in food intake in 10–12-week-old male mice (Supplementary Fig. 3) or in 16–17-week-old male mice (data not shown); however, O₂ consumption was significantly increased ($P < 0.05$; Fig. 2b). In addition, these mice showed increased locomotor activity and reduced respiration exchange ratios (Supplementary Fig. 3). When placed on a high fat diet (HFD), male *Agrp-Ires-cre*; *Vgat^{flox/flox}* mice had reduced body weight gain compared with controls (Fig. 2c), indicating that they had a resistance to diet-induced obesity (DIO) (Fig. 3a,c). Body composition analysis revealed that reduced body weight gain was solely because of reduced fat accumulation (Supplementary Fig. 3). The resistance to DIO was not the result of reduced food intake (Supplementary Fig. 3), which suggests that it was caused primarily by increased energy expenditure. To test this directly, we measured O₂ consumption in male mice during the transition from chow to HFD feeding. We observed a much greater increase in O₂ consumption in *Agrp-Ires-cre*; *Vgat^{flox/flox}* mice (Fig. 2d), indicating that these animals have increased HFD-induced thermogenesis.

AgRP neurons are a major target of ghrelin with respect to its effect on food intake¹¹. Ghrelin increased food intake in *Vgat^{flox/flox}* control mice, as previously reported¹¹. This effect, however, was greatly attenuated in *Agrp-Ires-cre*; *Vgat^{flox/flox}* mice in both males (Fig. 3a) and females (Fig. 3b), indicating that GABA release from AgRP neurons is an important mediator of ghrelin's stimulatory effect on food intake. To examine the possible effects on POMC neurons, we recorded inhibitory postsynaptic potentials in POMC neurons (Supplementary Methods). We visualized POMC neurons using a *Pomc-gfp* bacterial artificial chromosome transgene¹². Ghrelin significantly increased ($p < 0.05$) the frequency of inhibitory postsynaptic potentials recorded in POMC neurons of *Vgat^{flox/flox}* control mice, as previously reported¹³. This increase, however, was absent in *Agrp-Ires-cre*; *Vgat^{flox/flox}* mice (Fig. 3c,d), indicating that ghrelin-stimulated GABAergic input to POMC neurons is mediated by AgRP neurons.

Agrp-Ires-cre; *Vgat^{flox/flox}* mice are lean and resistant to DIO because of increased energy expenditure, suggesting that the normal function of GABA release from AgRP neurons is to restrain energy expenditure. This effect could be the result of GABAergic-mediated inhibition of POMC neurons, which are known to have a stimulatory effect on thermogenesis¹⁴. Notably, it appears that release of GABA from AgRP neurons, as opposed to AgRP and NPY⁴, is required for the regulation of energy expenditure. The alteration in body weight resulting from the disruption of GABA release from AgRP neurons is consistent with the 11% reduction in body weight and the lack of changes in feeding behavior of mice with neonatal ablation of AgRP neurons³. The marked attenuation of ghrelin-stimulated food intake in *Agrp-Ires-cre*; *Vgat^{flox/flox}* mice underscores the importance of GABA release from AgRP neurons in the orexigenic action of ghrelin, which is consistent with results from mice with AgRP neuron ablation¹⁵. The attenuation of ghrelin-stimulated food intake in *Agrp-Ires-cre*; *Vgat^{flox/flox}* mice is similar to that in mice with deleted AgRP or NPY⁴, suggesting that multiple signaling pathways downstream of AgRP neurons mediate ghrelin's orexigenic action. The absence of ghrelin-stimulated increases in IPSCs in POMC neurons of *Agrp-Ires-cre*; *Vgat^{flox/flox}* mice is consistent with POMC neurons mediating, indirectly via AgRP neurons, this orexigenic effect. In summary, our study demonstrates that GABA release from AgRP neurons is required for the regulation of energy expenditure and for ghrelin-stimulated food intake. We demonstrate

the usefulness of neuron-specific deletion of VGAT for studying the physiologic functions of GABAergic neurotransmission.

Supplementary Material

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Figure 1. Generation of mice lacking GABA release from AgRP neurons. All animal care and experimental procedures were approved by the Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. **(a)** Schematic diagram of the wild-type (WT) *Vgat* allele, the *Vgat^{flox/flox}* allele and the allele inactivated by Cre-mediated recombination. **(b)** Schematic diagram of the *Agrp-Ires-cre* allele. **(c)** The expression of Cre activity in the arcuate nucleus of *Agrp-Ires-cre* mice crossed with Cre-dependent [Au: Please clarify, is Z/EG the name of the mouse strain or is it the name of the transgene? I was unable to find Z/EG listed as a gene in Mouse Genomic Informatics or in Entrez Gene. Z/EG is an abbreviation of LacZ/EGFP, which is a line of transgenic mice. In these mice, the expression of LacZ/EGFP will be turned on with the presence of Cre recombinase.] GFP reporter Z/EG mice (immunostaining for GFP). **(d)** Summary of recordings for IPSCs and excitatory postsynaptic currents (EPSCs) from cultured AgRP neurons with autaptic synapses. To permit identification of AgRP neurons, we bred Z/EG GFP reporter mice to *Vgat^{flox/flox}* and *Agrp-Ires-cre*; *Vgat^{flox/flox}* mice. 3V, the third ventricle. Scale bar represents 10 μ M.

Figure 2.

Energy homeostasis in *Agrp-Ires-cre; Vgat^{lox/lox}* mice. (a) Body weight of *Vgat^{lox/lox}* mice and *Agrp-Ires-cre; Vgat^{lox/lox}* mice (males, $n = 14-19$ animals). (b) O₂ consumption of *Vgat^{lox/lox}* mice (7.02 ± 0.09 ml O₂ per h per g of lean mass) and *Agrp-Ires-cre; Vgat^{lox/lox}* mice (7.33 ± 0.10) ($n = 8$ each). O₂ consumption was measured using the Columbus Instruments Comprehensive Lab Animal Monitoring System and was normalized to lean mass. Lean mass was measured by dual-energy x-ray absorptiometry. (c) Body weight of *Vgat^{lox/lox}* mice and *Agrp-Ires-cre; Vgat^{lox/lox}* mice during HFD feeding ($n = 8$ each). (d) O₂ consumption of both *Vgat^{lox/lox}* mice and *Agrp-Ires-cre; Vgat^{lox/lox}* mice during the transition from chow to HFD feeding ($n = 6-9$). O₂ consumption rates were normalized to lean mass. The O₂ consumption rates of *Vgat^{lox/lox}* mice on 2 consecutive days on chow diet were 6.12 ± 0.07 and 6.16 ± 0.10 ml O₂ per h per g, respectively, and those on the following 2 consecutive days for HFD were 6.63 ± 0.08 and 6.63 ± 0.08 ml O₂ per h per g, respectively. The O₂ consumption rates of *Agrp-Ires-cre; Vgat^{lox/lox}* mice on 2 consecutive days on chow were 6.31 ± 0.07 and 6.46 ± 0.09 ml O₂ per h per g, respectively, and on 2 consecutive days on HFD were 7.02 ± 0.07 and 7.21 ± 0.08 ml O₂ per h per g, respectively. The net average increment of O₂ consumption in response to HFD was 0.45 ml O₂ per h per g in *Vgat^{lox/lox}* mice versus 0.70 ml O₂ per h per g in *Agrp-Ires-cre; Vgat^{lox/lox}* mice. Data are expressed as mean $\pm$ s.e.m. * $P < 0.05$.

Figure 3.

Ghrelin-induced food intake in *Vgat^{flox/flox}* mice and *Agrp-Ires-cre; Vgat^{flox/flox}* mice. **(a)** Food intake in 0.5-, 2- and 4-h time periods in male *Vgat^{flox/flox}* mice and *Agrp-Ires-cre; Vgat^{flox/flox}* mice after intraperitoneal administration of saline (S) or ghrelin (G, 500 μ g per kg of body weight, *n* = 6–10). For the 2-h period, food intake increased from 0.22 ± 0.05 g to 0.46 ± 0.06 g by ghrelin in *Vgat^{flox/flox}* mice versus 0.22 ± 0.05 g to 0.25 ± 0.05 g by ghrelin in *Agrp-Ires-cre; Vgat^{flox/flox}* mice. **(b)** Food intake in females using the same conditions as in **a**. For the 2-h period, food intake increased from 0.14 ± 0.04 g to 0.61 ± 0.06 g by ghrelin in *Vgat^{flox/flox}* mice versus 0.11 ± 0.05 g to 0.31 ± 0.05 g by ghrelin in *Agrp-Ires-cre; Vgat^{flox/flox}* mice. **(c)** IPSCs in POMC neurons of *Vgat^{flox/flox}* mice and *Agrp-Ires-cre; Vgat^{flox/flox}* mice. To permit identification of POMC neurons, *Pomc-gfp* transgenic mice were bred to *Vgat^{flox/flox}* and *Agrp-Ires-cre; Vgat^{flox/flox}* mice. Postsynaptic currents were recorded from GFP neurons in brain slices and IPSCs were isolated by eliminating glutamate-mediated EPSCs using d-APV and CNQX. **(d)** Summary of IPSC recordings in **c** (*n* = 8 each). Firing rate was increased from 1.44 ± 0.33 Hz to 2.60 ± 0.26 Hz by ghrelin in *Vgat^{flox/flox}* mice versus 1.04 ± 0.33 Hz to 1.23 ± 0.36 Hz in *Agrp-Ires-cre; Vgat^{flox/flox}* mice. d-APV and CNQX are glutamate receptor antagonists. Data are expressed as mean $\pm$ s.e.m. * *P* < 0.05.